Studies on Adenosine Triphosphate Transphosphorylases. Amino Acid Sequence of Rabbit Muscle ATP–AMP Transphosphorylase†

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ABSTRACT: The total amino acid sequence of rabbit muscle adenylate kinase has been determined, and the single polypeptide chain of 194 amino acid residues starts with *N*-acetylmethionine and ends with leucyllysine at its carboxyl terminus, in agreement with the earlier data on its amino acid composition [Mahowald, T. A., Noltmann, E. A., & Kuby, S. A. (1962) *J. Biol. Chem. 237*, 1138–1145] and its carboxyl-terminus sequence [Olson, O. E., & Kuby, S. A. (1964) *J. Biol. Chem. 239*, 460–467]. Elucidation of the primary structure was based on (1) tryptic and chymotryptic cleavages of the performic acid oxidized protein, (2) cyanogen bromide cleavages of the ^{14}C-labeled S-carboxymethylated protein at its five methionine sites (followed by maleylation of peptide fragments), and (3) tryptic cleavages at its 12 arginine sites of the maleylated ^{14}C-labeled S-carboxymethylated protein. Calf muscle myokinase, whose sequence has also been established, differs primarily from the rabbit muscle myokinase's sequence in the following: His-30 is replaced by Gln-30; Lys-56 is replaced by Met-56; Ala-84 and Asp 85 are replaced by Val-84 and Asn-85. A comparison of the four muscle-type adenylate kinases, whose covalent structures have now been determined, viz., rabbit, calf, porcine, and human [for the latter two sequences see Heil, A., Müller, G., Noda, L., Pinder, T., Schirmer, H., Schirmer, I., & Von Zabern, I. (1974) *Eur. J. Biochem. 43*, 131–144, and Von Zabern, I., Wittmann-Liebold, B., Untucht-Grau, R., Schirmer, R. H., & Pai, E. F. (1976) *Eur. J. Biochem. 68*, 281–290], demonstrates an extraordinary degree of homology. Thus, almost 93.8% of the structure of muscle-type myokinase has been conserved in these four mammalian species.

The isolation of rabbit muscle myokinase (ATP–AMP transphosphorylase) in crystalline form was first described by Noda & Kuby (1957a, 1963), who also determined several of its physicochemical properties (Noda & Kuby, 1957b). Adenylate kinase is an ubiquitous enzyme (Noda, 1973), and it has since been prepared in either crystalline or apparently homogeneous form and characterized from a variety of sources, which include rabbit (Noda & Kuby, 1957a,b, 1963; Kuby et al., 1978), calf (Kuby et al., 1978), porcine (Heil et al., 1974), carp (Noda et al., 1975), human (Thuma et al., 1972; Kuby et al., 1982, 1983), and rat muscle (Tamura et al., 1980) enzymes, the bovine liver mitochondria (Markland & Wadkins, 1966a,b) and human liver mitochondria (Kuby et al., 1982; Hamada et al., 1982) enzymes, the rat (Tamura et al., 1980; Criss et al., 1970; Sapico et al., 1972), calf (Kuby et al., 1978), porcine (Chiga & Plaut, 1960), and human liver (Kuby et al., 1982, 1983) adenylate kinases, and the enzyme from human erythrocytes (Tsuboi & Chervenka, 1975), rat hepatomas (Criss et al., 1974), rat brain (Pradhan & Criss, 1976), and bakers' yeast (Chin et al., 1967).

Recently, we have detected an aberrant adenylate kinase isoenzyme from the serum of patients with Duchenne muscular dystrophy (Hamada et al., 1981), which resembled isolated human liver mitochondria adenylate kinase (Hamada et al., 1982) in its kinetic, stability, and electrophoretic properties (Kuby et al., 1982; Hamada et al., 1981, 1982), but by immunological tests it appeared structurally to be a muscle type (Hamada et al., 1981, 1982; Kuby et al., 1982). It would be of interest from both a genetic and a pathogenetic standpoint to understand precisely which covalent alterations may have taken place in this human variant or mutant adenylate kinase isoenzyme.

The amino acid sequences have been deduced for the porcine muscle adenylate kinase (Heil et al., 1974) and one species of human muscle adenylate kinase, designated AK_1 (Von Zabern et al., 1976). The X-ray crystal structure of the porcine adenylate kinase (Schulz et al., 1974; Sachsenheimer et al., 1977; Pai et al., 1977) has been reported, and recently, the human and porcine muscle adenylate kinases were studied by ^{1}H nuclear magnetic resonance spectroscopy (Kalbitzer et al., 1982; Smith & Mildvan, 1982).

Recently, we have presented (Kuby et al., 1983) the amino acid analyses for the crystalline normal human liver, calf liver, and rabbit liver adenylate kinases and compared them with the normal muscle, calf muscle, and rabbit muscle myokinases. The liver types as a group and the muscle types as a group show a great deal of homology, but some distinct differences are evident between the liver and muscle enzyme groups, especially in the number of residues of His, Pro, and half-cystine and in the presence of tryptophan in the liver enzymes (Kuby et al., 1983).

The purpose of this paper is to present the covalent structures for the rabbit muscle myokinase and the calf muscle myokinase and to compare them with those published previously (Von Zabern et al., 1976; Heil et al., 1974). Eventually, these data for muscle-type adenylate kinases will lay the basis for a comparison of the structure of the liver-type adenylate kinases (Kuby et al., 1983) and for the aberrant Duchenne dystrophic adenylate kinase (Hamada et al., 1981, 1982; Kuby et al., 1982).

During the course of the determination of the amino acid sequences of the rabbit and calf muscle myokinase, certain relatively large peptide fragments (MT-I, residues 1–44, and MT-XII, residues 172–194) derived from the ^{14}C-labeled S-carboxymethylated protein became available for ligand binding studies. The unique ligand binding properties of these fragments, coupled with studies on a synthetic nonapeptide

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(residues 32–40 of the rabbit muscle enzymes), which included His-36 implicated by McDonald et al. (1975) as interacting with ATP, have led to a clearer picture of the two distinct substrate binding sites (but one catalytic site) for myokinase [see Hamada et al. (1979)]. A preliminary report has been presented (Kuby et al., 1976).

Experimental Procedures

Materials

Materials used as well as analyses, figures, and procedures pertinent to the isolation and sequencing of the various peptide fractions described in this paper are given in the supplementary material (see paragraph at end of paper regarding supplementary material). Figures and tables in the supplementary material are designated in the text with an S.

Rabbit muscle myokinase was isolated with the changes in procedure described in Kuby et al. (1978) and usually crystallized 3 times to a constant specific activity of ca. 2700 units/mg (pH-stat assay). The purity of this enzyme preparation as well as the calf myokinase has been established by a number of techniques, both physical and chemical [e.g., Kuby et al. (1978)].

Methods

Amino acid analyses followed that described for the rabbit muscle myokinase (Mahowald et al., 1962), for the rabbit muscle ATP-creatine transphorylase (Noltmann et al., 1962) and for the nucleoside diphosphokinase (Palmieri et al., 1973). Samples in sealed evacuated tubes (Pyrex) were hydrolyzed for periods of 20, 40, 70, and in some cases 140 h (e.g., to cleave Ile^{10}–Ile^{11}) in redistilled constant-boiling HCl. Analyses were conducted with a Spinco 120B amino acid analyzer modified to enhance the sensitivity to the nanomole range.

Half-cystine was determined either as *S*-(carboxymethyl)cysteine [after reduction with 2-mercaptoethanol and alkylation in 8 M urea with iodoacetic acid (Palmieri et al., 1973; Yue et al., 1967)] on the basis of Crestfield et al. (1963) or as cysteic acid, after performic acid oxidation by the procedure described by Moore (1963). Both amino acids, after acid hydrolysis, were referred to the measured value of leucine or norleucine as an internal standard. Negative values for tryptophan were repeatedly obtained after hydrolysis in 4 N $Ba(OH)_2$ for periods of 50–70 h at 100 °C in sealed evacuated Vycor glass tubes (Noltman et al., 1962) and analyses made with the developing system of Hugli & Moore (1972).

Cyanogen Bromide Cleavages. Cyanogen bromide cleavages (Gross & Witkop, 1961, 1962) were carried out as described below in 70% formic acid of the ^{14}C-labeled S-carboxymethylated protein at the five methionine sites, followed by maleylation (to render the peptide fragments soluble) and separation on 200-cm columns of Sephadex G-50 (fine) with NH_4HCO_3, pH 7.7, developers (Figure 13S).

(*a*) *^{14}C-Labeled S-carboxymethylated protein* was prepared as follows (Crestfield et al., 1963). A total of 2.0 μmol (M_r 21 400) of lyophilized (salt-free) 3 times crystallized rabbit muscle myokinase (preparation IW) was reduced with 2-mercaptoethanol under N_2 (gas phase) at 25 °C, for 30 min with the following final set of conditions: 8.0 M urea, 5.6×10^{-3} M EDTA,[1] 0.39 M Tris (Ac^-), pH 8.6, 0.111 M 2-mercaptoethanol, and at a protein concentration = 0.83 μmol/mL (or 1.67 μmol of –SH/mL, at 2.0 –SH/mol). This was followed with the alkylation step (under N_2, and in the dark, for 30 min) under the following final conditions: 8.0 M urea, 0.25 M Tris (Ac^-), pH 8.6, 5.0×10^{-3} M EDTA, 0.100 M 2-mercaptoethanol (calculated), 0.095 M iodoacetic acid (including 50 μCi of iodo[2-^{14}C]acetic acid, 54 mCi/mmol, Amersham), and 1.5 μmol of –SH/mL (16.0 mg of protein/mL) in a total additive volume of 2.64 mL.

The reaction was stopped by the addition of 5 μL of 2-mercaptoethanol; the contents were dialyzed (12 000 mol wt cut-off bags, A. H. Thomas) vs. 10 mM KCl and then vs. distilled water and lyophilized. Amino acid analyses revealed quantitative S-carboxymethylation at both thiols, with little loss, if any, in the five methionine residues (except traces of methionine sulfoxides). A radiospecific activity was estimated as 4.95×10^5 dpm per micromole of *S*-(carboxymethyl)cysteine for the two *S*-(carboxymethyl)cysteines/mol.

(*b*) *CNBr cleavage* followed in 70% formic acid, 10 mg/mL (0.5 μmol/mL protein concentration, at 25 °C, with a 50-fold molar excess of CNBr over its five methionines). The procedure was as follows. The above sample of S-carboxymethylated protein (42.3 mg = 1.98 μmol of protein, or 9.9 μmol of Met) was dissolved in 3.96 mL of 70% formic acid to a final protein concentration of 0.5 μmol/mL, followed by the addition of 0.30 mL of 2-mercaptoethanol (ca. 1.0 M). After 24 h at 25 °C to reduce any methionine sulfoxides to methionine, it was rapidly diluted with 80 volumes of ice-cold boiled redistilled H_2O and lyophilized, followed by redissolution and relyophilization. This was followed by the actual cleavage step. The sample in a small vial is dissolved (with a magnetic flea) in 3.96 mL of 70% formic acid, 0.20 mL of 2.5 M CNBr in 70% formic acid (freshly prepared) is added, and the sample is covered with Saran-Wrap and stoppered. The solution is stirred (magnetically) slowly at 25 °C for 16 h, rapidly diluted with 80 volumes of ice-cold redistilled water, as before, and lyophilized. After dissolution in a small volume of ice-cold redistilled water and relyophilization, the sample is ready for maleylation. The larger fragments tend to aggregate and to precipitate, and to render them soluble in aqueous solution, maleylation has been found to be a convenient technique.

(*c*) *Maleylation* of the ^{14}C-labeled S-carboxymethylated CNBr fragments followed with a 40-fold excess of maleic anhydride over its 20 lysyl residues, at pH 9.0. The procedure is as follows. The above sample (assumed to be 1.98 μmol of lysine residues) is suspended in 6.6 mL of 0.2 M borate (Na^+), pH 9.0, 25 °C to a concentration of 6 μmol of lysine residues/mL and chilled in an ice bath. A calculated amount of maleic anhydride (Sigma) is added, equal to a 40-fold excess over the ϵ-NH_2 groups (i.e., $40 \times 39.6 = 1584$ μmol or 155.3 mg), and divided into six equal aliquots. After the addition of each aliquot (with stirring) of solid maleic anhydride, 2 N NaOH is added to maintain the pH at 9 (±0.2); 5–10 min is allowed for the reaction between aliquots. Finally, after the last addition of maleic anhydride, 30 min (with stirring) is allowed for complete reaction and to maintain the pH at 9. The solution should be clear at this point (if not, it is clarified by centrifugation); it was then transferred immediately to a 2.3×170 cm (160-cm bed height) column of G-50 (fine) and developed (by ca. 1 m of hydrostatic head) with 0.10 M NH_4HCO_3 (MCB, freshly prepared), pH 7.7 [Figure 13S; cf. Schmidt & Hirs (1974)]. Except for the 69-residue fragment (residues 126–194), there is reasonable separation according to size of the 60-residue fragment (2–61), the 45-residue fragment (81–125), the 14-residue fragment (62–75), and the

[1] Abbreviations: TPCK, 1-(tosylamido)-2-phenylethyl chloromethyl ketone; dansyl, 5-(dimethylamino)-1-naphthalenesulfonyl; PTH, 1-phenyl-2-thiohydantoin; DFP, diisopropyl phosphorofluoridate; S-CmCys, *S*-(carboxymethyl)cysteine; S-S_2O_3, sulfenyl thiosulfate; TNB, 5-thio-2-nitrobenzoate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

5-residue fragment (76–80). The acylhomoserine is obscured under the large UV-absorbing area due to excess maleic acid, etc. Each area, including the front edge of the first radioactive area (which contained largely residues 126–194), was pooled and lyophilized. Prior to automated Edman degradations (see below), samples were demaleylated by treatment with 2 mL of 0.2 M pyridine (acetic acid), pH 3.16 at 40 °C., for 72 h (Evans et al., 1974). Ten volumes of cold distilled water (ice cold) was added and lyophilized. In Table IIIS are summarized the analytical data obtained on the isolated cyanogen bromide fragments.

Tryptic Cleavages of the Maleylated ^{14}C-Labeled S-Carboxymethylated Protein. This technique was modeled after the maleylated–tryptic digestion approach of Butler et al. (1969) on the ^{14}C-labeled S-carboxymethylated protein. A typical case is as follows: 55.6 mg (2.6 μmol) of reduced and alkylated protein (with iodo[^{14}C]acetic acid) was dissolved in 6 mL of 4 M guanidinium chloride in 0.2 M borate, pH 9.0, and a 40-fold molar excess (27 mg) of solid maleic anhydride relative to the 20 ϵ-NH_2 groups added in six equal aliquots with stirring (magnetic), while maintaining the pH with 2 N NaOH at 9 (±0.5) in an ice bath, as above. After the last addition, another 30 min was allowed to elapse with the pH maintained at 9 (ice bath).

The sample was desalted by dialysis (12 000 mol wt cut-off bags) against 10 mM NH_4HCO_3, pH ca. 8.9, and lyophilized. From the initial specific radioactivity of the alkylated enzyme (1.14×10^6 dpm/μmol), ca. 93% recovery of protein was obtained (2.41 μmol) or, for 12 Arg/mol, 28.9 μmol of arginine residues. Digestion at 30 °C with TPCK-trypsin followed with the aid of a pH-stat radiometer TTT1a titrator with a pH A630T scale expander, SBR2C Titrigraph, and ABU1b autoburette. The conditions were as follows: the protein concentration (initial) was ca. 7.5 mg/mL, dissolved in 1 mM KCl; after the pH was adjusted to 8.5 at 30 °C with 0.104 N NaOH, 1% (mol/mol) of trypsin was added to initiate the very rapid reaction; near the end of the reaction, another 1% (mol/mol) of trypsin was added. Within 1 h, 12.3 bonds had been cleaved (blank corrected), compared to the theoretical of 12; the reaction was terminated by the addition of 5 μL of 0.1 M phenylmethanesulfonyl fluoride (in dry 2-propanol), and the reaction mixture was lyophilized. This was followed with fractionation on a Sephadex G-50 (fine) column (2.5 × 172 cm) [cf. Schmidt & Hirs (1974)] at 25 °C, developed with 0.1 M NH_4HCO_3, pH 7.7 (see Figure 14S);[2] each of the peaks (except peak II, residues 1–44) was further purified on DEAE-Sephadex A-25 columns (with a linear gradient in NH_4HCO_3), e.g., Figures 4–9S,[2] or on Dowex 50-X2 (with an exponential gradient in pyridine–acetic buffers), e.g., Figures 1S, 11S, and 12S.[2]

The 5-thio-2-nitrobenzoate protein (TNB protein) (preparation 3) was prepared as follows. A total of 36.5 mg (1.71 μmol) of rabbit muscle myokinase (preparation I-V) was dissolved in 5.0 mL of 0.2 M borate–4.0 M guanidine hydrochloride, pH 9.0, and a 5.1-fold excess of DTE (8.78 μmol) was added. After 1 h at 25 °C, a 123-fold excess of DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] or 210 μmol of solid DTNB was added in several small aliquots with the pH maintained with 2 N NaOH (by pH-stat) at ca. 9.0. After 1 h, the reaction appeared to be complete, and the TNB protein solution was chilled in a ice bath and was *maleylated* directly in the pH-stat vessel, with ca. a 46-fold excess of solid maleic anhydride over ϵ-amino groups (ca. 1600 μmol), added in 6 equal aliquots of ca. 26 mg each time with the pH maintained at ca. 9. After ca. 1 h, the reaction was over, and the reaction mixture was dialyzed vs. 2 times 500 mL of 0.1 M NH_4HCO_3, pH 8.42 at 5 °C, and then vs. 2 times 500 mL of 10 mM NH_4HCO_3, pH 8.3 at 5 °C, and lyophilized.

Trypsin digestion followed at pH 8.5 with TCPK-trypsin (1% w/w) at a myokinase protein concentration initially of ca. 9 mg/mL. After the reaction was over and the trypsin activity was inhibited with 5 μL of 1 M phenylmethanesulfonyl fluoride, it was applied directly to a Sephadex G-50 (fine) column (2.5 × 176 cm) developed with 0.1 M NH_4HCO_3, pH 7.7.[2] Some fragments were purified further, after demaleylation, by chromatography on Dowex 50-X2 with pyridine–HOAc developers (Figure 10S).[2] Table IVS[2] provides an analytical summary of the maleylated tryptic peptides studied.

The sulfenyl thiosulfate (S-S_2O_3) protein (preparation 4) was prepared as follows. To 37 mg (1.76 μmol) of myokinase, at 4 °C, in 5 mL of 0.1 M $NaHCO_3$–5 mM DTE (pH 8), was added guanidine hydrochloride to 2.0 M, followed by the addition of a 300-fold excess of solid $Na_2S_4O_6 \cdot 2H_2O$ divided into two aliquots (of ca. 80 mg each time), with the pH maintained at 7.7–8.0. After a total time for reaction of ca. 3 h, the sample was dialyzed vs. 10 mM $NaHCO_3$ and lyophilized. The sample to be *maleylated* was dissolved in 6 mL of ice-cold 4 M guanidinium chloride in 0.2 M borate, pH 9.0, and a 40-fold excess (over lysine residues per mole) of maleic anhydride (solid) was added in six equal aliquots, with the pH maintained at ca. 9 (at 4 °C) with 2 N NaOH (as described above). After dialysis vs. 1 mM NH_4OH and lyophilization, ca. 31.2 mg or 1.46 μmol of protein was recovered, which was then digested with *TPCK-trypsin*, as described above for the ^{14}C-labeled S-carboxymethylated cysteine maleylated protein; followed by separation on Sephadex G-50 fine, 2.5 × 172 cm, developed with 0.1 M NH_4HCO_3 (Figure 2S).[2]

Tryptic Digestions of the Performic Acid Oxidized Protein. Performic acid oxidation of the two thiols and five methionine residues was performed as follows. The performic acid solution was prepared according to Hirs (1956). A total of 1.0 mL of H_2O_2 (30%, Mallinkrodt, A. R.) was added to 19.0 mL of formic acid (97–100% MCB), mixed, placed at 25 °C for 2 h, and chilled. A total of 293 mg (13.7 μmol) of salt-free lyophilized rabbit muscle myokinase (preparation I-K, 3 times crystallized) was dissolved in 6 mL of cold formic acid plus 1.0 mL of redistilled methanol (anhydrous), and 11.5 mL of performic acid was added (a 30-fold molar excess) and placed in a −10 °C bath for 2.5 h. The contents were diluted with 30-fold volume of ice-cold redistilled water and relyophilized. This was repeated twice more with ca. 95% recovery of protein. A total 12.8 μmol of performic acid oxidized protein was dissolved in the pH-stat vessel with 8.6 mL (or 1.5 μmol/mL protein) of 10 mM NaCl–1 mM $CaCl_2$; when the pH was adjusted to an end point of 8.0 with 0.196 N NaOH at 30 °C, 1% (on a molar basis) of TPCK-trypsin was added to initiate the reaction, followed later (5 h) by another 1% (mol/mol) of trypsin; a third addition of trypsin (1% on a molar basis) was added after ca. 25 bonds had been cleaved, and the reaction was terminated (27.5 h) when 30–32 bonds were cleaved (theoretical = 12 Arg + 20 Lys = 32). After termination of the reaction with 5 μL of 0.1 M DFP, the contents were lyophilized. The sample was subjected to peptide mapping (Figure 15S)[2] according to Yue et al. [1967; cf. Katz et al. (1959)] and then chromatographed on Dowex 50-X2 (Figure 16S)[2] according to Yue et al. (1967), with pyridine–acetic acid developers [see Table IS; cf. Kimmel et al. (1962)]. Fractions were pooled, lyophilized, and, if necessary, further purified

[2] See paragraph at end of paper regarding supplementary material.

on Dowex 1-X2 columns with developing systems (see Table IIS) similar to those of Schroeder et al. (1962) or by paper electrophoresis or paper chromatography, in over-all yields of 40–75%, in general.[2] Table VS[2] summarizes the data on all the tryptic peptides isolated and analyzed.

Chymotryptic cleavages were conducted, similarly, on the performic acid oxidized protein,[2] and Table VIS[2] provides a summary of those chymotryptic peptides analyzed.

Sequencing Methodology. Manual sequencing of the isolated tryptic peptides was conducted by the procedure of Edman, by a modification of the adaptation of Konigsberg & Hill (1962). The subtractive approach, coupled with identification of the PTH-amino acid by thin-layer chromatography (Stahl, 1965), or alkaline conversion of the PTH derivatives to the free amino acids (Africa & Carpenter, 1966) was employed. In addition, dansylation of the amino-terminal residue (Gray, 1972) prior to each cycle of the Edman degradation and separation of the dansyl amino acids on polyamide sheets (Gray, 1972) were utilized. The data for each peptide is given in the supplement material[2] but are summarized in Schemes IS–IVS.[2]

Carboxyl terminal residues (for chymotryptic peptides, especially) were estimated by hydrazinolysis (Yue et al., 1967) and by carboxypeptidase B digestions (or B followed by carboxypeptidase A digestions). Amino peptidase M digestions were employed for glutamine and asparagine estimations, where practicable.

Automated Edman degradations were performed with a Beckman Model 890C modified with cold trap, with 0.1 M quadrol as the coupling buffer and with use of the Beckman program 121078. The spinning cup was pretreated with polybrene (Aldrich Chemical Co.) according to Hunkapillar & Hood (1978), and five cycles were run in the presence of lysylglycine prior to the loading of the large peptide fragments. Fractions recovered from each cycle were automatically converted in 0.2 N methanolic HCl from the thiazolinone derivatives to the phenylthiohydantoins with a Sequemat P-6 converter. The PTH derivatives were identified by high-pressure liquid chromatography according to Margolies & Brauer (1978) with a μ-C_{18} reverse-phase column (Waters) at 55 °C, with a linear gradient of 27–50% acetonitrile in 20 mM sodium acetate buffer, pH 5.0, and with use of a Waters SISP-790A instrument.

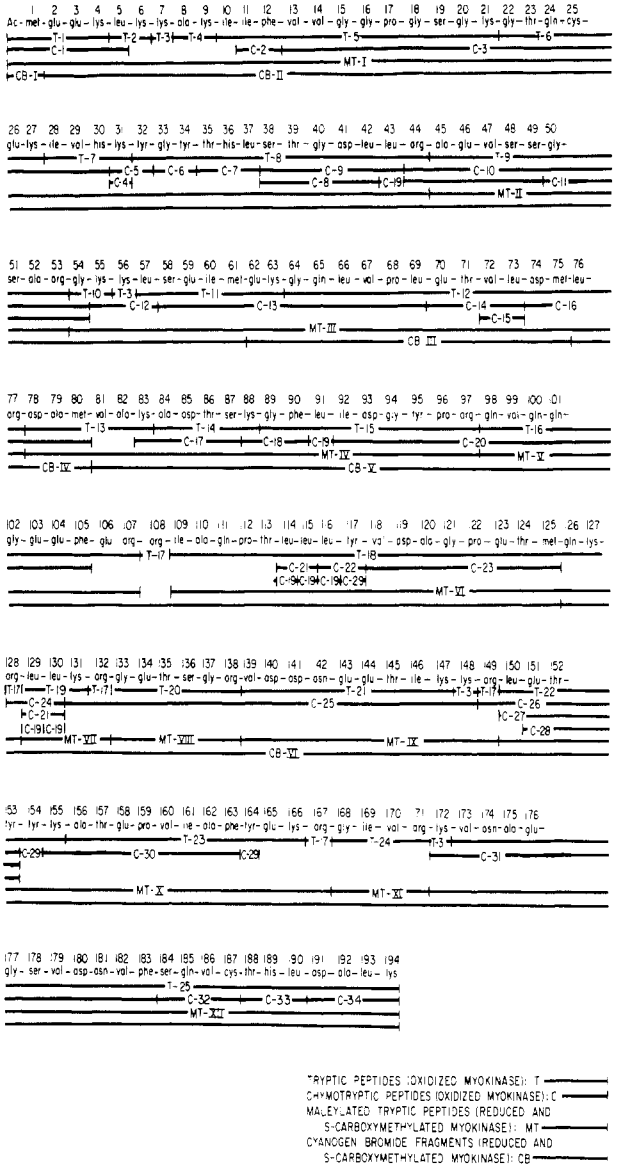
Some detailed procedures, figures, tables, and analyses are presented in the supplementary material;[2] but, see Schemes IS–IVS[2] for summaries of those peptides sequenced by the various methods, and see Tables IIIS–VIS[2] for a summary of analytical data obtained on each of the peptides isolated and sequenced.

Results and Discussion

The strategy behind the structural analyses may be presented as follows. Rabbit muscle myokinase contains no tryptophan (Table VIIS);[2] consequently, performic acid oxidation proved to be a good starting point for the tryptic and chymotryptic cleavages, the former for the required methionine (5) overlaps and the latter for the required arginine (12) overlaps.

The ^{14}C-labeled S-carboxymethylated maleylated myokinase could then be cleaved at its 12 arginine sites with trypsin, and the isolation and sequences of the resulting fragments after demaleylation provided the necessary overlaps at the 20 lysyl residues. Similarly, cleavages of the ^{14}C-labeled S-carboxymethylated myokinase with cyanogen bromide yielded fragments cleaved at the five methionine sites, which contained the final overlaps for the tryptic and chymotryptic peptides.

Scheme I: Determination of the Amino Acid Sequence of Rabbit Muscle ATP–AMP Transphosphorylase (Myokinase)



In this last case, it proved necessary to maleylate relatively large fragments, to render them soluble and to facilitate their chromatographic separation.

In Scheme I, the results are presented of the determination of all the sequences completed in the structural analyses. In Scheme I, the peptide nomenclature follows that according to the cleavage agents by which they were obtained. Thus, T for tryptic, C for chymotryptic, MT for maleylated tryptic peptides, and CB for cyanogen bromide fragments (in this case methionine is replaced by homoserine). One notes that there are sufficient overlaps to provide some confidence in the total amino acid sequence of rabbit muscle myokinase.

In Table VS[2] are summarized the amino acid compositions, yields, and residues locations of the isolated tryptic peptides obtained from the performic acid oxidized myokinase (cf. Figure 16S).[2] The peptide map presented in Figure 15S,[2] acted as a check on the isolation of all the tryptic peptides, and the coordinates of each isolated peptide[2] were determined and compared with the original map.

In Scheme IS[2] are summarized those sequences determined on all the tryptic peptides isolated from the performic acid oxidized rabbit muscle myokinase. The symbols given in Scheme IS describe the methodology employed in the sequential degradation, and in the supplementary material the

necessary quantitative data are provided for each technique, as well as the methods of final isolation of each peptide.[2] Peptide T-1 proved to have no free amino terminus, and after hydrazinolysis, acethydrazide was isolated. Exhaustive digestion with carboxypeptidase C liberated Lys, followed by Glu_2, and after purification and hydrazinolysis of the peptide, which contained only $MetSO_2$, acethydrazide and $MetSO_2$ were obtained. Its sequence accordingly was *N*-Ac-Met-SO_2-Glu-Glu-Lys(COOH) or residues 1–4 (inclusive) of the rabbit muscle myokinase. The relatively large 22 residues long tryptic peptide T-25 proved to be at the carboxyl end (residues 173–194), and this peptide highlights an interesting point: viz., almost one-third (or seven) of all the lysine residues (total of 20) may be found clustered at or near the amino terminus (from residues 1–44), whereas only two lysine residues are to be found at the carboxyl end (residues 172–194). Further, a triplet sequence of Thr-His-Leu is repeated twice in the molecule at residues 34–36, near the amino end, and at 188–190, near the carboxyl end.

Similarly, Tables IIIS, IVS, and VIS[2] provide the amino acid compositions, yields, and residue locations of the chymotryptic peptides of the performic acid oxidized rabbit muscle myokinase (Table VIS),[2] of the tryptic cleavage fragments of the maleylated ^{14}C-labeled S-carboxymethylated (or S-thiosulfated or S-thionitrobenzoated) rabbit muscle myokinase (Table IVS),[2] or of the maleylated cyanogen bromide cleavage fragments of the ^{14}C-labeled S-carboxymethylated rabbit muscle myokinase (Table IIIS).[2] It should be noted that alkylation at the two thiols (residues 25 and 187) with 2-iodo[^{14}C]acetate provided a convenient radioactive marker for these two residues and their associated peptide fragments and facilitated greatly the isolation of these particular peptide fragments, especially MT-I and MT-XII (Scheme I and IIIS), the two fragments that have been shown to possess unique ligand and substrate binding properties (Hamada et al., 1979). But unfortunately, the S-carboxymethyl peptides do not show any catalytic properties, and in an effort to provide a reversible blocking group to the thiols, residues 25 and 187, the thiosulfate and thionitrobenzoate groups were introduced. These derivatized peptides also provided in some cases improved resolution in their chromatographic behavior (see Figures 2S, 3S, and 10S).

In Schemes IIS–IVS[2] are summarized the sequence data for the chymotryptic peptides, the tryptic cleavage fragments of the maleylated ^{14}C-labeled S-carboxymethylated rabbit muscle myokinase, and the maleylated cyanogen bromide cleavage fragments of the ^{14}C-labeled S-carboxymethylated rabbit muscle myokinase.

In Table VIIS a comparison is drawn of the amino acid composition estimated originally by Mahowald et al. (1962) and that estimated from the overall covalent structure given in Scheme I. Originally, Mahowald et al. (1962) had listed 11 arginine residues per 21447 g of enzyme, since 11.54 was found and it was rounded off to 11, rather than 12, as given by the structure. Residue 108, an arginine, was discovered only by the cyanogen bromide cleavage technique (CB-V fragment), yielding the only Arg^{107}-Arg^{108} sequence in the structure. In Kuby et al. (1978) therefore, 11 arginines are listed and 193 total residues, instead of 12 arginines and 194 total residues. There are actually 9 isoleucines as estimated by the structure, but only 8.44 could be estimated even after 140 h of 6 N HCl hydrolysis at 110 °C. In actual fact, 70 h of acid hydrolysis would not cleave the peptide bond between Ile^{10}-Ile^{11} in the tryptic peptide (T-5), but it was readily cleaved by digestion with amino peptidase M. In all other cases, there is an exact agreement, and even the eleven amides that were originally estimated could be distributed among the three asparagine and eight glutamine residues obtained from the structure. In the last column, a comparison is drawn with the calf muscle myokinase (Kuby, S. A., et al., unpublished results), whose total covalent structure has also been determined. The homology between these two enzymes, the rabbit and calf muscle myokinase, is striking. Although these two enzymes differ by only seven residues, it is of interest to note that the calf enzyme may act as a powerful antigen in the rabbit, from whose serum a potent anti-calf muscle myokinase or anti-human muscle myokinase may be isolated (Kuby et al., 1978). The sequence of the calf muscle myokinase differs primarily from that of the rabbit muscle myokinase in the following: His-30 in the rabbit protein is replaced by Gln, Lys-56 by Met-56, Ala-84 by Val, and Asp-85 by Asn. Interestingly, all are essentially conservative replacements. It is noteworthy that there are two histidine residues in the calf, His-36 and His-189, vs. three in the rabbit, His-30, His-36, and His-189, and only subtle differences have been found in their steady-state kinetics (Hamada & Kuby, 1978) and in their substrate binding properties (Hamada et al., 1979). But His-36 and His-189 have been conserved, apparently, in both of their respective nucleotide binding sites, i.e., for $MgATP^{2-}$ and for AMP^{2-} (Hamada et al., 1979).

The covalent structures of the four muscle-type adenylate kinases, rabbit, calf, porcine, and human, whose sequences have now been determined, are shown in Scheme II [see Heil et al. (1974) and Von Zabern et al. (1976)]. Their homology is striking. Residues 8, 30, 45, 56, 84, 85, 98, 100, 110, 126, 127, and 181 are the only loci where replacements have been introduced in all four mammalian muscle-type adenylate kinases. Thus, almost 93.8% of the structure of muscle-type myokinase has been conserved over a significant period of evolutionary development. On the other hand, each of the liver types (or mitochondrial types; Hamada et al., 1982) of the adenylate kinases of calf, human, and rabbit differ by only 10–20% in their amino acid compositions when compared with their respective muscle-type (or cytoplasmic) counterpart (Kuby et al., 1983), whereas, again, the liver types also showed striking homology when compared as a group. It would now, therefore, be of interest to establish the covalent structures of the liver-type adenylate kinases to compare them with the muscle-type structures. A comparison of the kinetic and molecular properties of the calf isoenzymes (Kuby et al., 1978; Hamada et al., 1978, 1979) and of the human isoenzymes (Hamada et al., 1982; Kuby et al., 1983), especially the unusual fact that Ap_5A [P^1,P^5-bis(adenosyl)pentaphosphate] acts as a potent inhibitor of the rabbit and calf muscle types (Kuby et al., 1978), but is far less active as an inhibitor of the calf and human liver types (Kuby et al., 1978; Hamada et al., 1981), has led to the postulate (Hamada & Kuby, 1978; Hamada et al., 1981) that these two enzymes differ structurally, in that the liver-type isoenzyme may already exist in solution in a partially fixed or preferred conformation so as to facilitate phosphoryl group transfer from $MgATP^{2-}$ to AMP^{2-}.

Finally, mention should be made of the sequences of peptide fragments MT-I and MT-XII, derived from the head and tail of the rabbit muscle myokinase molecule, respectively (Schemes I and IIIS). As already noted, both fragments, interestingly, contain an identical tripeptide sequence, viz., Thr^{35}-His^{36}-Leu^{37} and Thr^{188}-His^{189}-Leu^{190}. Both His-36 and His-189 have been the subject of extensive NMR investigations in the porcine muscle myokinase (McDonald et al., 1975;

Scheme II: Amino Acid Sequence of Rabbit Muscle ATP-AMP Transphosphorylase (Myokinase) Compared with Calf Muscle Myokinase, Porcine Muscle Adenylate Kinase (Heil et al., 1974), and Human Muscle Adenylate Kinase (Von Zabern et al., 1976)

```
            1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16  17  18  19  20  21  22  23  24
RABBIT:   AC-MET-GLU-GLU-LYS-LEU-LYS-LYS-ALA-LYS-ILE-ILE-PHE-VAL-VAL-GLY-GLY-PRO-GLY-SER-GLY-LYS-GLY-THR-GLN-
          |-------------------------------------------- MT-I ---------------------------------------------
CALF:                                  -ALA-
HUMAN:                                 -THR-
PORCINE:                               -SER-

          25  26  27  28  29  30  31  32  33  34  35  36  37  38  39  40  41  42  43  44  45  46  47  48  49
RABBIT:   CYS-GLU-LYS-ILE-VAL-HIS-LYS-TYR-GLY-TYR-THR-HIS-LEU-SER-THR-GLY-ASP-LEU-LEU-ARG-ALA-GLU-VAL-SER-SER-
          ---------------------------------------------------------------------------|
CALF:                         -GLN-                                                   -ALA-
HUMAN:                        -GLN-                                                   -SER-
PORCINE:                      -GLN-                                                   -ALA-

          50  51  52  53  54  55  56  57  58  59  60  61  62  63  64  65  66  67  68  69  70  71  72  73  74
RABBIT:   GLY-SER-ALA-ARG-GLY-LYS-LYS-LEU-SER-GLU-ILE-MET-GLU-LYS-GLY-GLN-LEU-VAL-PRO-LEU-GLU-THR-VAL-LEU-ASP-
CALF:                             -MET-
HUMAN:                            -LYS-
PORCINE:                          -MET-

          75  76  77  78  79  80  81  82  83  84  85  86  87  88  89  90  91  92  93  94  95  96  97  98  99
RABBIT:   MET-LEU-ARG-ASP-ALA-MET-VAL-ALA-LYS-ALA-ASP-THR-SER-LYS-GLY-PHE-LEU-ILE-ASP-GLY-TYR-PRO-ARG-GLN-VAL-
CALF:                                        -VAL-ASN-                                               -GLN-
HUMAN:                                       -VAL-ASN-                                               -GLU-
PORCINE:                                     -VAL-ASP-                                               -GLU-

          100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118  119 120 121 122 123 124
RABBIT:   GLN-GLN-GLY-GLU-GLU-PHE-GLU-ARG-ARG-ILE-ALA-GLN-PRO-THR-LEU-LEU-LEU-TYR-VAL- ASP-ALA-GLY-PRO-GLU-THR-
CALF:     GLN-                           -ARG-ILE-ALA-
HUMAN:    GLN-                           -ARG-ILE-GLY-
PORCINE:  LYS-                           -ARG-ILE-GLY-

          125 126 127 128 129 130  131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149
RABBIT:   MET-GLN-LYS-ARG-LEU-LEU- LYS-ARG-GLY-GLU-THR-SER-GLY-ARG-VAL-ASP-ASP-ASN-GLU-GLU-THR-ILE-LYS-LYS-ARG-
CALF:         GLN-LYS
HUMAN:        THR-ARG
PORCINE:      THR-LYS

          150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175
RABBIT:   LEU-GLU-THR-TYR-TYR-LYS-ALA-THR-GLU-PRO-VAL-ILE-ALA-PHE-TYR-GLU-LYS-ARG-GLY-ILE-VAL-ARG-LYS-VAL-ASN-ALA-
                                                                                              |---------------
CALF:
HUMAN:
PORCINE:

          176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194
RABBIT:   GLU-GLY-SER-VAL-ASP-ASN-VAL-PHE-SER-GLN-VAL-CYS-THR-HIS-LEU-ASP-ALA-LEU-LYS.
          ------MT-XII--------------------------------------------------------------|
CALF:                        -ASN-
HUMAN:                       -GLU-
PORCINE:                     -ASP-
```

Smith & Mildvan, 1982) and in human muscle myokinase (Kalbitzer et al., 1982), and most important, His-36 has been implicated as interacting with ATP (McDonald et al., 1975). The substrate binding properties of these unusual peptide fragments, MT-I and MT-XII, have been described (Hamada et al., 1979), and these studies lent support to the hypothesis of Rhodes & Lowenstein (1968), who had deduced kinetically that two separate and distinct binding sites exist (a) for the magnesium complexes of the nucleotide substrates, $MgATP^{2-}$ and $MgADP^{-}$ (e.g., at the head), and (b) for AMP^{3-} and ADP^{3-} (e.g., at the tail of the myokinase molecule) [cf. Noda (1962, 1973)] but with one overall catalytic site. It is, of course, necessary to postulate a conformational change that may bring both substrate binding sites into juxtaposition (Hamada et al., 1979), in the case of the muscle-type adenylate kinases. Such a conformational change has been implicated by NMR studies as a result of the binding of Ap_5A to human muscle adenylate kinase (Kalbitzer et al., 1982). Following the conformational change, phosphoryl group transfer should then be facilitated so as to allow catalysis of the overall reaction $MgATP^{2-} + AMP^{2-} \rightleftharpoons MgADP^{-} + ADP^{3-}$ (Hamada & Kuby, 1978). Further studies are now in progress on the liver-type adenylate kinase covalent structures and on the relationship of the covalent structures of the muscle types to their substrate interactions.

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Supplementary Material Available

Analyses, figures, tables, schemes, and procedures pertinent to the isolation and sequencing of the various peptide fractions described in this paper (85 pages). Ordering information is given on any current masthead page.

Registry No. Adenylate kinase (rabbit muscle reduced), 89618-27-9; adenylate kinase (ox muscle reduced), 89618-26-8; adenylate kinase, 9013-02-9.

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